

FACTORS ASSOCIATED WITH CONIDIAL PRODUCTION
AND GROWTH OF *CERCOSPORA PERSONATA* (BERK.
& CURT.) ELL. & EV. CAUSING TIKKA DISEASE
OF GROUNDNUT (*ARACHIS HYPOGAEA* L.)

By

R. P. PURKAYASTHA

Department of Botany, University of Calcutta,
India.

Attempts have been made to revive the sporulating character of *Cercospora personata*, a causal organism of tikka disease of groundnut. Incidentally, vegetative growth, production of conidia per colony and conidial characteristics have also been studied. Of the eleven media tested, yeast extract agar appears to be the best medium for the growth while carrot agar is most suitable for conidial production of the test fungus. The size and septation of conidia are markedly affected by the culture media used. The differences between conidial lengths on different media are statistically significant. The conidia obtained from in vitro cultures are usually longer than those isolated from the host species grown under natural conditions. Length of light period directly stimulates both sporulation and mycelial growth but continuous darkness inhibits sporulation and also retards the rate of growth. Light treatment is more effective in increasing conidial production provided the cultures are exposed to continuous light after a few days incubation in complete darkness. The combination of trace elements, viz., Cu, Zn, Mn and Fe upto 1 $\mu\text{g/ml}$ concentration of each with basal medium favours vegetative growth. If Zn is omitted the medium itself slightly accelerates conidial production. Definite ratios of glucose and KNO_3 used in the medium exhibit no significant difference between dry weights of mycelia. Selective sub-culturing technique seems to be an useful method for regaining spores in the culture of *C. personata*.

INTRODUCTION

Cercospora personata usually sporulates in suitable media under favourable conditions but difficulty is often encountered in obtaining spores after a few months in repeated sub-cultures. It gradually loses its ability to produce spores eventually forming an aberrant growth habit. Shanta (1956) stated that the sporulating character of the said fungus could be revived if single spore cultures were prepared. It has been observed that the growth rate of monoconidial cultures of *C. personata* is extremely slow and therefore it is hardly possible to get an adequate supply of spores within a short period for experimental purposes. Further, a complete disappearance of spores from cultures during the progress of work is rather frustrating to workers. Hence, it was thought worthwhile to find out a suitable technique by which the sporulation could be induced or increased in vitro. During preliminary investigation, the effects of various media, light period, trace elements and different ratios of glucose and KNO_3 on conidial production and mycelial growth of the said fungus have been studied. Subsequently, a technique for selective sub-culturing has also been devised in order to regain the sporulating character.

MATERIALS AND METHODS

Cercospora—infected leaves were collected from the experimental garden of the University College of Science, Calcutta during November, 1965. Three leaves were treated with 0.1% mercuric chloride for 2-3 minutes, washed with sterile water, transferred to a moist filter paper in a sterilized Petri-dish, incubated at room temperature (21°-23°C) and diffused light for 6 days. The conidia were then transferred aseptically from the lesion areas to several carrot agar slants and incubated as described. After three weeks, the cultures were examined and the characteristics of the conidia and the growth habit were compared with the pure culture of *C. personata* which was kindly supplied by Prof. T. S. Sadasivan, University of Madras, and found to be identical. Subsequently, monoconidial cultures were made by the dilution plate culture method. To prepare inoculum for the growth and sporulation experiments, 3 weeks old cultures in conical flasks (250 ml.) were flooded with a known volume of water, shaken and filtered through sterile muslin. The spores with small hyphal fragments were washed by centrifugation and eventually suspended in sterile water. The culture flasks (100 ml.) containing 20 ml medium in each were inoculated with 0.2 ml. of mycelial and spore suspension and spread uniformly as far as possible on the surface of the media. Three replicates were usually employed for each treatment. The inoculated flasks were incubated at 23°-26°C and in continuous light from three fluorescent tubes (40 watt each) at a distance of 2 ft from the bottom of the flasks. After a desired period of incubation, the cultures were examined and their characteristics noted. At the end of the experimental period, the solid media in the culture flasks were melted and strained through muslin. The loss in weight due to spores, hyphal fragments and certain soluble cell components during straining and melting of agar was negligible. As all the replicates were similarly treated, the results might therefore, be comparable. The mycelial colonies collected in small aluminium cups of known weight were then kept in the hot air oven at 60°C for 72 hours. Finally, they were cooled in a desiccator and weighed. For spore counting, 10 colonies from each replicate flask were collected at random and each colony was put in a drop of lactophenol—cottonblue on a glass slide, stirred with an inoculating needle for 1 minute and subsequently the colony was removed; the remaining spores and few hyphal fragments were covered with a square cover glass and the number of spores counted under the microscope.

EXPERIMENTAL RESULTS

Culture media

Various media including coconut milk (unheated) agar and autoclaved groundnut leaf extract agar were tested under identical conditions of light, temperature 20°-22°C and humidity. As the test fungus grows better in solid than in liquid medium the following media were supplemented with 2% agar.

Table 1. Length, width and septation of conidia, sporulation, diameter of colony, mycelial growth and change of pH of culture media after 4 weeks growth of *C. personata*.

Medium	pH		*Average diameter of colony (mm)	Range of length of conidia (μ)	**Mean conidial length with S.E.	Range and mean conidial width (μ)	Range of septation	Dry wt. of mycelia (mg.)	Spores/colony
	Initial	Final							
Onion agar	5.8	5.5	1	24.99—57.12	36.65 \pm 1.60	5.4—7.1 (6.8)	1—5 (2)	159.20	73
Carrot agar	5.8	6.3	1	21.42—67.83	41.30 \pm 2.18	5.3—8.9 (7.5)	1—6 (3)	120.05	103
Coconut milk (unheated) agar	5.2	5.6	1	21.42—71.40	40.46 \pm 2.12	5.4—7.1 (6.8)	1—4 (3)	50.30	92
Oatmeal agar	5.8	5.6	1	28.56—60.69	43.43 \pm 1.71	5.4—8.9 (7.1)	2—6 (3)	19.55	13
Groundnut leaf extract agar	6.0	7.2	<1	24.99—60.69	39.27 \pm 1.21	5.4—7.1 (6.8)	1—3 (3)	10.15	38
Potato-dextrose-agar (PDA)	5.8	7.5	1	21.42—67.83	38.03 \pm 2.28	5.4—8.9 (6.4)	2—8 (3)	99.70	34
Potato-dextrose-coconut milk (autoclaved) agar	5.8	7.5	1	17.85—49.98	32.61 \pm 1.41	5.4—8.9 (6.8)	1—3 (2)	114.85	18
Malt agar	5.6	4.2	1	32.13—71.40	46.65 \pm 2.09	5.4—7.1 (6.8)	1—5 (3)	39.70	66
Basal medium	5.3	5.4	<1	24.99—57.12	38.79 \pm 1.55	5.4—7.1 (7.1)	2—6 (3)	9.80	7
Basal medium with yeast extract (Difco) agar	5.4	6.5	1	21.42—49.98	34.51 \pm 1.56	5.4—7.1 (6.8)	1—5 (3)	184.35	18
Glucose-peptone-casamino acid agar	5.3	6.2	1	17.85—60.69	34.27 \pm 2.05	5.3—7.1 (6.8)	1—5 (2)	75.10	53

*average of 60 colonies/treatment ; **average of 30 conidia/treatment ; ***No. in bracket indicates the number of septa in majority (60—50%) of the conidia examined.

(i) Onion (ii) carrot (iii) coconut milk (50 ml coconut milk/50 ml water) (iv) oatmeal (v) groundnut leaf extract (10 gm fresh wt. of leaves/100 ml water) (vi) potato-dextrose (vii) potato-dextrose and coconut milk (viii) malt (1.5%), (ix) basal medium (glucose, 1%, peptone, 0.2%, KH_2PO_4 , 0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%) (x) basal medium with 0.1% yeast extract (Difco) and (xi) glucose-peptone-casamino acid medium (glucose, 1%; peptone, 0.2%; KH_2PO_4 , 0.15% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05%; KCl , 0.5%; NaNO_3 , 0.6% and caseinhydrolysate 0.3%) were selected for this investigation. As the pH of the test media was found to be between 5.2–6, no further adjustment was thought necessary prior to inoculation. This range of pH is satisfactory for the growth of the said fungus. The flasks containing media were inoculated and incubated as described. After 4 weeks, the cultures were examined and mycelial growth, average diameter of colonies, change of pH of media, sporulation, length, width and septation of conidia on various media were noted. The results are given in Table 1.

The results suggest that carrot agar is an ideal medium for sporulation of *C. personata* although coconut milk (unheated) agar is also suitable for conidial production under similar experimental conditions. For mycelial growth, both yeast extract and onion agar appear to be highly favourable. Beside rate of growth and sporulation, the length of conidia is significantly affected (Table 2) by the culture media used. The conidia produced on malt agar attain maximum length (mean 46.65μ) while minimum length (mean 32.61μ) has been noted from those on potato-dextrose-coconut milk agar. The number of septa in a conidium

Table 2. Effect of various media on conidial length of *C. personata*—Analysis of variance.

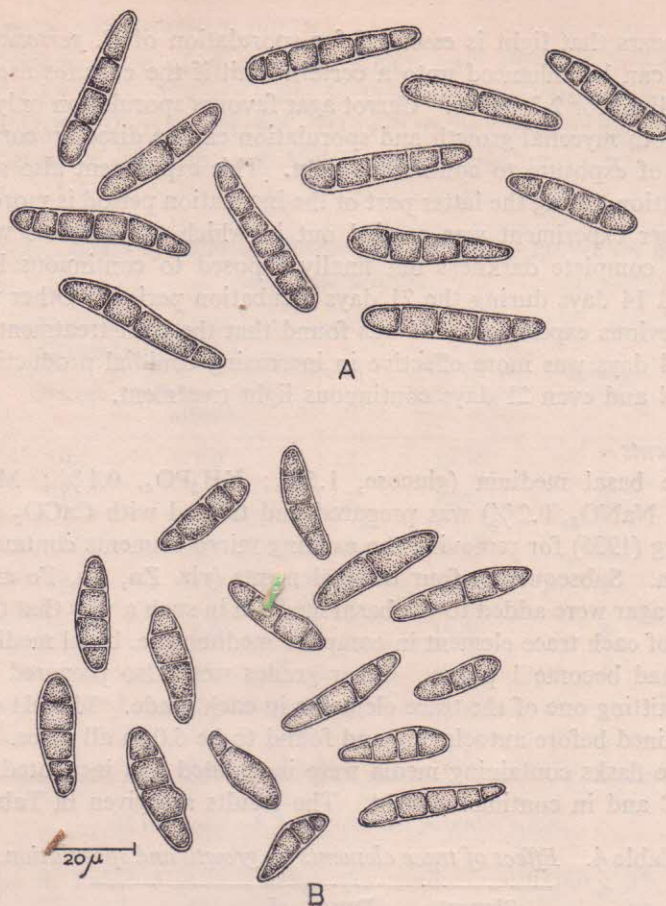
Source of variation	D.F.	S.S.	M.S.S.	'F'*** value
Media	10	5368.9594	536.9	5.1
Replicates	29	2164.4947	74.6	
Error	290	30177.9957	104.1	
Total	329	37711.3498		

***significant at 5% and 1% levels.

usually varies between 1-8 but triseptate conidia are most commonly found. It would be worthwhile to mention here that the conidia obtained from culture media are generally longer (length $21-68\mu$) than the conidia isolated from the necrotic regions (Length $19-39\mu$) of the leaves of host plants grown under natural conditions (TEXT-FIG. 1). Another interesting feature is that, except onion, oatmeal and malt agar, the pH of the remaining culture media has shown a trend towards alkalinity after 4 weeks' growth of the test species.

Light period

In order to evaluate the role of light on growth and sporulation, the flasks containing carrot agar media were inoculated and exposed to different periods (first 2, 7 and 14 days) of continuous light and temperature at $25 \pm 1^\circ\text{C}$. Subsequently, these were transferred to complete darkness. In all cases the total in-

Text-fig-1. (A-B)—*C. personata*

A. Conidia in culture ; B. Conidia from lesion areas

cubation period was 21 days. One set of cultures was kept in darkness throughout the 21 days while another set was maintained under continuous light for a similar period. The results are given in Table 3.

Table 3. Effect of different periods of light on growth and sporulation.

Length of ** light/dark period (days)	Average No. of spores/colony	Dry wt. of mycelia (mg) av. of 3 replicates
<i>Light</i>		
*2	0	14.7
*7	2	16.8
*14	8	20.3
21	146	29.5
<i>Dark</i>		
21	0	9.5

*Total incubation period was 21 days in all cases ; **120 W from 3 fluorescent tubes at a distance of 2' ft from the bottom of the flask.

It appears that light is essential for sporulation of *C. personata*. Conidial production can be enhanced upto a certain limit if the cultures are exposed to continuous light for 2-3 weeks. Carrot agar favours sporulation only in presence of light. Both mycelial growth and sporulation can be directly correlated with the period of exposure to continuous light. This experiment also suggests that the illumination during the latter part of the incubation period is more critical.

Another experiment was carried out in which the cultures were initially kept under complete darkness but finally exposed to continuous light for the last 2, 7 and 14 days during the 21 days incubation period. Other factors were same as previous experiment. It was found that the light treatment for the last 2, 7 and 14 days was more effective in increasing conidial production than the first 2, 7 14 and even 21 days continuous light treatment.

Trace elements

The basal medium (glucose, 1.5% ; KH_2PO_4 , 0.1% ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% and NaNO_3 , 0.2%) was prepared and treated with CaCO_3 as suggested by Steinberg (1935) for removing the existing micro elements contaminants from the medium. Subsequently four trace elements (viz. Zn, Cu, Fe and Mn) and 2.5% clear agar were added to the basal medium in such a way that the final concentration of each trace element in complete medium (i.e. basal medium + 4 trace elements) had become 1 p.p.m. Other grades were also prepared in a similar way but omitting one of the trace elements in each grade. The pH of the media was determined before autoclaving and found to be 6.0 in all cases. After sterilization, the flasks containing media were inoculated and incubated for 4 weeks at 23°-26°C and in continuous light. The results are given in Table 4.

Table 4. Effect of trace elements on growth and sporulation.

Element omitted	Dry wt. of mycelium (mg)	Spores/colony
None	19.15	7
Zinc	14.65	26
Copper	17.45	3
Manganese	10.20	9
Iron	14.80	13

From the data it is clear that *C. personata* grows well when all the four trace elements are added to the medium. If manganese is omitted the growth becomes very poor. It is probable that either the combination of Cu, Zn, and Fe up to a concentration of 1 p.p.m. of each in the medium is not favourable for the growth of the test fungus or manganese is necessary for better growth. Results also indicate that basal medium supplemented with Cu, Mn, and Fe may stimulate sporulation while lack of copper may slightly reduce the production of conidia.

Ratio of glucose and potassium nitrate

The concentrations of glucose and KNO_3 were adjusted to get the desired ratios (1 : 1, 1 : 2, 2 : 1, 3 : 1, 4 : 1 and 5 : 1) of the said substances in the medium containing KH_2PO_4 (0.05%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05%) and clear agar (2.5%). The maximum levels of glucose and KNO_3 in the medium were 4% and 1% respectively.

Table 5. *Effect of ratio of glucose and potassium nitrate on growth and sporulation.*

Concentrations (%)		Ratio	Dry wt. mycelia (mg)	Spores/colony
Glucose	Potassium nitrate			
0.5	1.0	1 : 2	19.76	7
1.0	1.0	1 : 1	16.76	8
2.0	1.0	2 : 1	19.63	11
3.0	1.0	3 : 1	23.00	4
4.0	1.0	4 : 1	19.86	8
1.5	0.3	5 : 1	19.66	6
2.5	0.5	5 : 1	19.86	4
4.0	0.8	5 : 1	19.83	5

pH of media—5.2 ; incubation period 4 weeks.

Results in Table 5 indicate that the medium containing 3% glucose and 1% KNO_3 is most favourable for the growth of *C. personata*. When equal proportions (1 : 1) of these two substances are added to the medium, the fungus grows at a slower rate. Statistical analysis, however, shows that the differences between the dry weights of mycelia due to variation in ratios of glucose and KNO_3 of the medium are not significant.

Selective sub-culturing technique

A sporulating colony from a spore-losing culture was transferred aseptically to a little quantity of sterile water, crushed with a sterile forceps and subsequently inoculated into the culture flasks containing carrot agar by flooding the surface of the media with a few drops of prepared suspension and incubated under conditions as stated earlier. It was found that average conidia production per colony increased to a considerable extent within 2-3 weeks. The experiment was repeated several times with similar results obtained in every case.

This technique seems to be preferable in maintaining the adequate number of spores in the cultures for a longer period provided this selective sub-culturing is done at regular intervals.

DISCUSSION

C. personata, a causal pathogen of the tikka disease of groundnut sporulates on a wide range of culture media when it is first isolated from the host but it gradually loses its ability to produce spores in culture irrespective of the media used. The reduction of conidial production can not be checked by using only mycelial or a mixture of mycelial and spore suspension as inocula. Although it has been suggested by Shanta (1956) that the sporulating character could be revived by single spore cultures other difficulties are also encountered which have been pointed out earlier in this paper. The selective sub-culturing technique which has been used in the present investigation, appears to be more convenient, less time consuming and suitable for large scale spore production.

Apart from the renewal of the sporulating character, the results of the present study also reveal the effect of some cultural factors associated with the vegetative growth and conidial characteristics of the species concerned. Of the several media tested, carrot agar appears to be an ideal medium for sporulation. It seems likely that carotenoid compounds or their derivatives might stimulate conidial production under certain conditions. It is also evident from the results that there is no consistent relationship between mycelial growth and sporulation on the eleven media tested. The variation in the lengths of conidia of *C. personata* like several other fungi has been reported by previous workers (Jenkins, 1938 ; Vasudeva, 1963). In the present study, the lengths of conidia show significant differences statistically on various culture media incubated under identical condition of light, temperature and humidity. Maximum and minimum mean lengths (46.65 μ and 32.61 μ) of conidia have been recorded on malt agar and potato dextrose—coconut milk (autoclaved) agar respectively. It is of interest to note that the conidia obtained from culture media are usually longer than the conidia isolated from the host plant grown under natural conditions. The formation of smaller conidia on lesion areas might be due to the influence of environmental factors including moisture contents or presumably due to any other constituent of the host plant which is thermolabile because longer conidia are not uncommon on autoclaved groundnut leaf extract agar. Data show that light is an essential factor for both sporulation and vegetative growth. The duration of the light period can be directly correlated with the rate of growth and conidial production. It has also been found that light treatment is more effective when cultures are illuminated after a few days incubation in complete darkness. It is not unreasonable to speculate that light is probably necessary at the time of spore formation and as such more spores are obtained when 2-3 weeks old cultures are exposed to continuous light.

The results with trace elements strongly suggest that the combination of all four trace elements (viz. Cu, Zn, Mn and Fe) upto 1 μ g/ml concentration of each might increase the vegetative growth to some extent but have very little effects on sporulation. However, the remarkable effect of one or more trace elements on conidial production can not be ruled out if a long range of concentration is taken into account. It has been observed that when Mn is omitted in the medium,

the mycelial growth becomes very poor. Similar result has also been reported by Steinberg (1945) in case of *Aspergillus niger*.

All attempts to increase conidial production of *C. personata* were not met with equal success. However, the selective sub-culturing technique, light treatment under favourable temperature and selection of carrot agar medium might help in regaining or increasing conidial production in artificial cultures. Calpouzos (1954) has succeeded in controlling the sporulation of *Cercospora musae* and Jones (1958) has been able to isolate a stable sporulating strain of *C. kikuchii* from soybean by selective sub-culturing but the techniques used by the said workers are different from that used in the present investigation. The success in regaining spores by isolating a sporulating colony from a large number of non-sporulating mycelial colonies suggests that a genetic factor might be involved in conidial production by *C. personata*. Further research, is therefore necessary to elucidate the underlying mechanism.

The author is indebted to Prof. T. S. Sadasivan, Director and Head of the Department of Botany, University of Madras for kindly supplying the culture of *C. personata*. Thanks are also due to Dr. S. N. Banerjee, Reader in Botany, Calcutta University for providing laboratory facilities and to the Council of Scientific and Industrial Research, Govt. of India for financial assistance.

REFERENCES

- Calpouzos, L. (1954). Controlled sporulation of *Cercospora musae* Zimm. in pure culture. *Nature* **173**, 1084-1085.
- Jenkins, W. A. (1938). Two fungi causing leaf spot of peanut. *Jour. Agric. Res.*, **56**, 317-332.
- Jones, J. P. (1958). Isolation of a sporulating strain of *Cercospora kikuchii* by selective sub-culturing. *Phytopath.* **48**, 287-288.
- Shanta, P. (1956). Isolation of *Cercospora personata*, its sporulation and growth in pure culture. *Proc. Ind. Acad. Sc.*, **44B**, 271-275.
- Steinberg, R. A. (1935). Nutrient solution purification for removal of heavy metals in deficiency investigation with *Aspergillus niger*. *Journ. Agric. Res.*, **51**, 413-424.
- Steinberg, R. A. (1945). A dibasal (minimum salt, maximum yield) solution for *Aspergillus niger*, acidity and magnesium optimum. *Plant Physiol.*, **20**, 600-608.
- Vasudeva, R. S. (1963). *Indian Cercosporae*. *Ind. Coun. Agric. Res. publ.*

(Accepted for publication 7th September 1968)